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## Abstract

Cell contraction and membrane blebbing are evolutionarily conserved events that occur during the execution phase of apoptosis. Several members of the TNF-ligand superfamily, which are associated with the promotion of a number of pathological processes, including inflammation and cancer are also capable of inducing membrane blebbing in some cell types. Most of these ligands are transmembrane bound but can be shed from the cell surface through proteolytic processing where soluble ligands can act as antagonists, as in the case of FAS ligand, or agonists, as with TNF-α. Here we provide evidence that the matrix metalloproteinase, MMP-3/stromelysin-1 induces rapid membrane blebbing in serum starved or cyclohexamide-treated MCF10A human breast epithelial cells. MMP-3-mediated membrane blebbing is associated with reorganization of the actin cytoskeleton, upregulation of both p53 and p38 MAP kinase activity, and loss of cell surface E-cadherin. A broad-spectrum MMP inhibitor completely abolishes these reactions. To understand the signaling cascade initiated by MMP-3, we asked whether factors downstream of TNF-superfamily signaling were involved. We show that inhibitors against JNK and caspase-3, and RNAi reduction of MKK7, a known activator of JNK inhibit membrane blebbing. Moreover, stable expression of a dominant negative FADD (dnFADD), a downstream effector of several TNF superfamily ligands, renders MCF10A cells resistant to membrane blebbing. Together these findings indicate that MMP-3 induces cell membrane blebbing through a TNF-superfamily signaling pathway and provides an impetus to further explore this protease in inflammation and cancer.

## Table of Contents

INTRODUCTION

The family of Matrix metalloproteinases (MMPs) consists of secreted and membrane-bound zinc endopeptidases. Our lab has focused research on a particular MMP named stromelysin-1 (MMP-3) which functions in the cell's microenvironment through degradation of extracellular matrix and shedding of cell surface proteins[1]. In breast cancer, MMP-3 is inadvertently highly expressed in tumor cells and the surrounding stroma when compared to normal breast tissue [2]. Previously, we reported that over-expression of MMP-3 is associated with E-cadherin cleavage and epithelial to mesenchymal transition (EMT) in cultured mammary epithelial cells, and also leads to an increase in epithelial apoptosis, a mammary inflammatory response, precocious mammary gland differentiation with hyperplasia, and mammary tumorigenesis in transgenic mice [3,4,5]. The mechanism(s) behind these varied MMP-3-"driven" phenotypes remains largely undiscovered. In efforts to elucidate a mechanism behind MMP-3-induced tumorigenesis we recently have found that overexpression of MMP-3 in mouse mammary epithelial cells to leads to the expression of an alternatively spliced form of Rac1 called Rac1b. Rac1b overexpression subsequently generates an increase in cellular reactive oxygen species (ROS). The generation of ROS led to both the expression of an EMT-inducing transcription factor Snail and causes oxidative damage to DNA and subsequent genomic instability[6]. Although these findings point to a novel mechanism of MMP-3-induced genomic instability it should be noted they were derived in a cell line absent in p53 function.

In normal cells, the tumor suppressor, p53, functions as a key regulator of DNA replication, DNA repair, cell cycle progression, and apoptosis, largely through its ability to act as a transcription factor [6]. Events that compromise p53 function may predispose tissues to neoplastic transformation [7], while those that activate p53 function may induce cell cycle arrest or apoptosis. Loss of function is most often associated with either p53 inactivating mutations, or mutations in, or deregulation of, key p53 regulatory proteins. These regulatory proteins include kinases, histone acetyl transferases, sumoylases and ubiquitinases, all of which directly modify p53 to provide orchestrated regulation of p53 protein levels and function. Activation of p53 is most often associated with genomic or environmental stress and is conserved evolutionary response seen from yeast to humans. Whereas much research has focused on the intracellular proteins that regulate p53 function, very few studies have been directed toward extracellular proteins as regulators of p53 function.

Our results have indicated that at high MMP-3 concentrations (>800 nM) p53 protein increases and becomes activated in both human and mouse mammary epithelial cells. The following report outlines these findings and the progress toward understanding the mechanisms behind MMP-3-dependent regulation of the tumor suppressor protein p53.

BODY (All figures are in Appendix I)

In my previous annual report I reported that at concentrations greater than 800 nM, MMP-3 induces upregulation of p53 protein levels in both human and mouse mammary epithelial cells (Fig. 1 and 2). To determine whether other MMPs have a similar capability I examined two gelatinases (MMP-2, and -9 that have distinct substrates compared to MMP-3) and found that both were unable to induce p53 protein levels (Fig. 1B). Moreover, the non-specific protease

trypsin did not alter p53 protein levels (data not shown). These results suggest that MMP-3 is a unique protease capable of influencing p53 protein levels, a novel finding for the MMP field and the secreted protease field in general.

Since p53 protein levels were changing in response to MMP-3 it was important to determine whether p53 function was also being altered. We focused our attention on p21, a known p53 target gene that functions predominantly as a growth arrest factor. As depicted in Fig. 1B, the amount of p21 protein mirrors that of p53 indicating that MMP-3 is likely affecting p53 function.

Since we have recently reported that MMP-3 can induce ROS[6], we asked whether the generation of ROS contributes to p53 upregulation. Our findings, using the ROS scavenger DMSO, suggest that indeed ROS generation by MMP-3 is necessary for p53 upregulation (Fig. 2). It is important to note that DMSO did not alter MMP-3-induced cell-cell detachment and cell rounding, suggesting it does not directly inhibit MMP-3 activity (Fig 3).

After approximately 6 hrs, at concentrations of MMP-3 that leads to upregulation of p53 protein cells begin to detach from each other and round up. This phenotype is seen in both mouse and mammary epithelial cells (Fig. 3 and 4). Importantly, cell-cell detachment is inhibited by a broad spectrum MMP inhibitor (Fig. 4D). In the human breast epithelial cell line MCF10A, MMP-3 induces membrane blebbing (Fig. 4E), a process that is often associated with cell apoptosis.

The fact that MMP-3 induces blebbing led us to speculate whether a TNF-α-like pathway was being stimulated, as these pathways are the primary mediators of this phenotypic response. This could occur through shedding of cell surface ligands within this superfamily. In fact TNF-α can be cleaved by MMP-3 in solution [7].

The TNF-α pathway can either induce differentiation/proliferation or apoptosis depending on the state of the cell [8]. Specifically, if translation is halted or slowed, using CHX or serum starvation, respectively, TNF-α induces cell blebbing and apoptosis in the majority of cell types, while in the absence of these conditions no such phenotypes are seen. This parallels our findings with MMP-3-induced cell blebbing as seen in Fig. 5, where only in CHX or serum starved cells do we see MMP-3 induce cell-cell detachment and cell blebbing.

In MCF10A cells (Fig. 6) and in EPH4 cells (data not shown) examination of p53 protein by immunocytochemistry and confocal imaging revealed a large increase in cytoplasmic nuclear p53 and accumulation of p53 sub-nuclear foci when treated with high levels of MMP-3.

Suppression of blebbing was seen when a JNK inhibitor (JNK is a kinase activated when the apoptotic arm of these pathways are elicited) was added to MMP-3 treated cells (Fig. 7), while other inhibitors such as those against PI3K, and MAPK, failed to do so (data not shown).

Another pathway that is often upregulated in a TNF-α-like response is p38, similar to JNK, p38 is a stress activated kinase. Investigations using immunocytochemistry and immunoblotting reveal that this pathway is upregulated in response to high levels of MMP-3 (Fig. 8). Upregulation of p38 may also contribute to increased levels of p53 since p53 is a substrate of p38.

Upstream of JNK and p38 activation is FADD activation and FADD activation is downstream of receptor binding by several TNF-α receptor family members. I have found that MCF10 clones that have stable expression of a dominant negative FADD are completely resistant to high levels of MMP-3 (Fig. 9).

Using RNAi, I down-regulated the levels of MKK7, a kinase downstream of FADD and upstream of JNK and p38 activation. Two MCF10A clones that exhibited a decrease in MKK7 (data not shown), were completely resistant to MMP-3-mediated blebbing and cell-cell detachment (Fig. 10)

We hypothesized that MMP-3-mediated shedding is releasing a TNF-α receptor family member that can now bind to its cognate receptor. Using RT-PCR we examined which members of the TNF-alpha superfamily are expressed on MCF10A cells. Our findings were surprising in that several members are found expressed in these cells (Fig. 11). Several of these ligands are thought to be specific for the leukocyte lineage and have yet to be found expressed in mammary epithelial cells. Since membrane blebbing and activation of FADD involves receptors that possess death domains we focused our efforts on three membrane-tethered ligands that bind to such receptors (TNF-alpha, FAS, and TRAIL). RT-PCR revealed that of these three candidate ligands only TRAIL was expressed in MCF10A cells (Fig. 12).

Since activation of TNF-α-like pathways have often been found lead to upregulation of p53 protein we have begun to ask whether this pathway is responsible for MMP-3-mediated upregulation of p53. We first asked whether p53 transcription was being upregulated by MMP-3. Our results indicate that this is not the case – treatment with MMP-3 does not increase p53 message when compared with untreated or sham treated controls (Fig. 13). These findings have led us to now focus on posttranslational modifications of p53 as a mechanism for its upregulation.

## FUTURE EXPERIMENTS TO DETERMINE HOW MMP-3 INDUCES ACTIVATION OF A TNF-α-LIKE PATHWAY.

- Determine if blocking TRAIL association with its receptor suppresses MMP-3-induced cellular phenotypes.
- Examine phosphorylation sites on p53 after MMP-3 treatment.
- Use inhibitors against JNK, p38 to determine whether these pathways are responsible for p53 upregulation.
- Address whether MMP-3 induces a complete apoptotic response.
- Determine if these MMP-3-mediated phenotypes are found in other mammary epithelial cell lines.

## KEY RESEARCH ACCOMPLISHMENTS

- MMP-3, not MMP-2 or MMP-9, can influence p53 protein levels/function in mammary epithelial cells. General proteases like trypsin do not influence p53.

- At MMP-3 concentrations >800 nM (high), p53 protein levels increase and cellular apoptotic-associated events become evident, such as cell-cell detachment, cell shrinkage, and cellular blebbing.

- At high concentrations p53 protein accumulates in sub-nuclear compartments and p21 protein levels increase substantially.

- Upregulation of p53 is not through increased transcription of the p53 gene.

- Cell blebbing is inhibited by MMP inhibitors and JNK inhibitors.

- Upregulation of p53 by MMP-3 is suppressed by reactive oxygen scavengers.

- MMP-3 activity leads to activation of the stress activated kinase p38.

- At high concentrations of MMP-3, cellular phenotypes are suppressed by inhibiting pathways associated with a TNF-α-like response.

- Down regulation of MKK7, a kinase that functions downstream of FADD activation, suppresses MMP-3-mediated blebbing.

- TRAIL is expressed on MCF10A cells and is a possible candidate ligand that may be activated by MMP-3.

## REPORTABLE OUTCOMES

A proportion of this work was presented at the American Society of Cell Biologists (ASCB, 2003, San Francisco, CA) and at the DOD Era of Hope (Philadelphia, PA). The abstracts as published are given in Appendix II. As well efforts to better understand MMP-3-mediated cellular phenotypes were recently published. See attached abstract.

## CONCLUSIONS

In response to MMP-3 both mouse and human mammary epithelial cells undergo cell-cell detachment and cell rounding. However, the human cells, MCF10A, also exhibit membrane blebbing that can only be induced when these cells are serum starved or pretreated with cyclohexamide. The TNF-pathways also induce membrane blebbing under these conditions. Using various methods to inhibit multiple factors involved in TNF signaling we have found that indeed MMP-3 activates a TNF-like response. We hypothesize this is through MMP-3-mediated shedding of a TNF ligand, thereby allowing it to bind to its cognate receptor. In an effort to find a candidate ligand activated by MMP-3 an analysis of the expression of all the TNF-ligands was performed. In this screen we found TRAIL to be a strong candidate as its receptor contains a death domain capable of activating many of the factors we found to be necessary for MMP-3-induced blebbing. We are now addressing whether MMP-3 sheds off TRAIL to activate FADD,

MKK7, JNK, p38 and apoptosis, as well as the initiating event in generation of ROS and upregulation of p53 protein and activity. These findings would represent a novel pathway elicited by MMP-3 and provide insight into how MMP-3 overexpression may determine cell fate.

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APPENDIX I

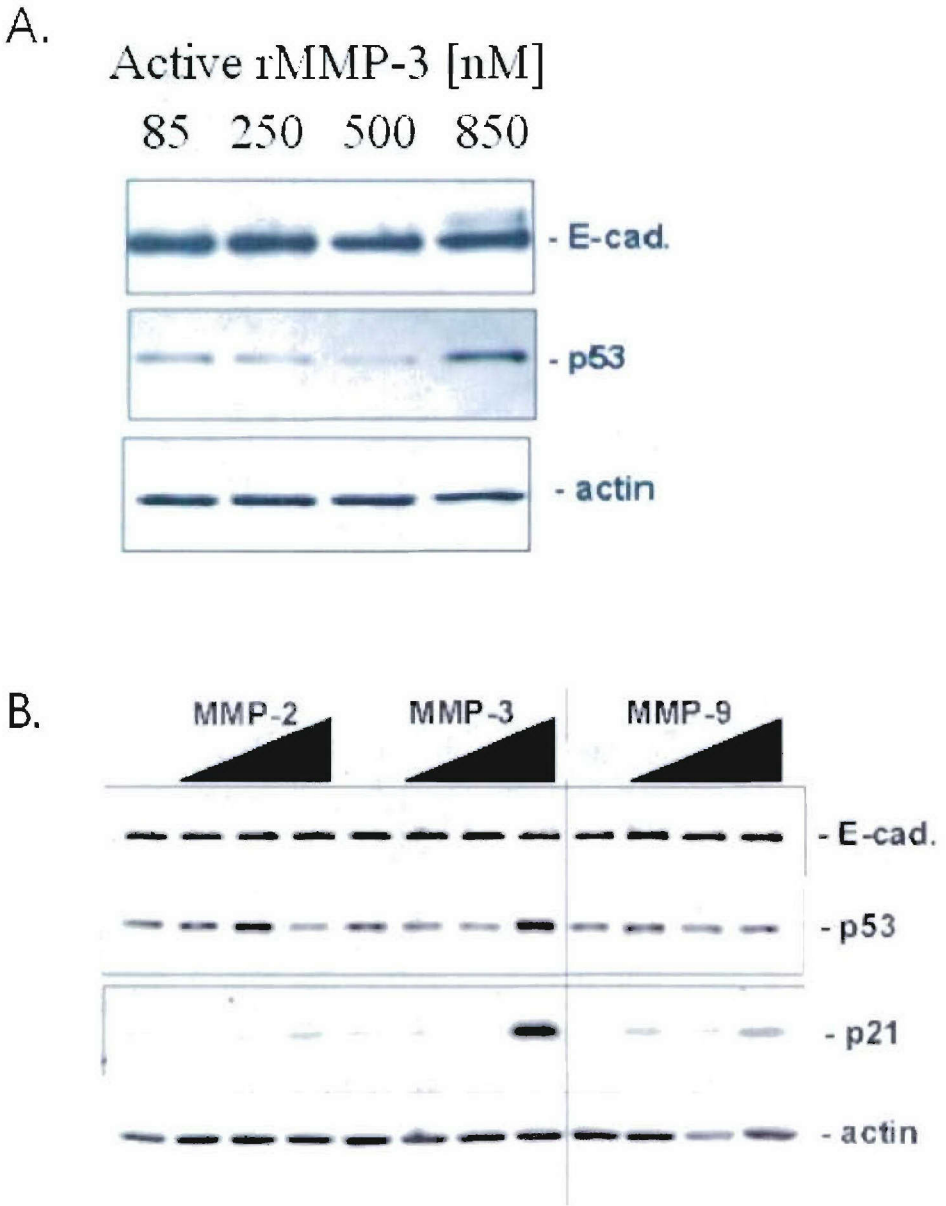



Fig 1A. MCF10A cells treated with various concentrations of recombinant active MMP-3. At all concentrations except 850 nM, p53 protein decreases as MMP-3 increases. However, at 850 nM MMP-3, p53 protein increases markedly.

Fig. 1B. MCF10A cells treated with active MMP-2, -3, and -9. Only MMP-3 treated cells exhibit p53 modulation. P21, a target gene of p53, mirrors the expression of p53 in MMP-3 treated cells. MMP-2 and –9 treated cells do not have any apparent dose dependent regulation of p53 or p21.

2.

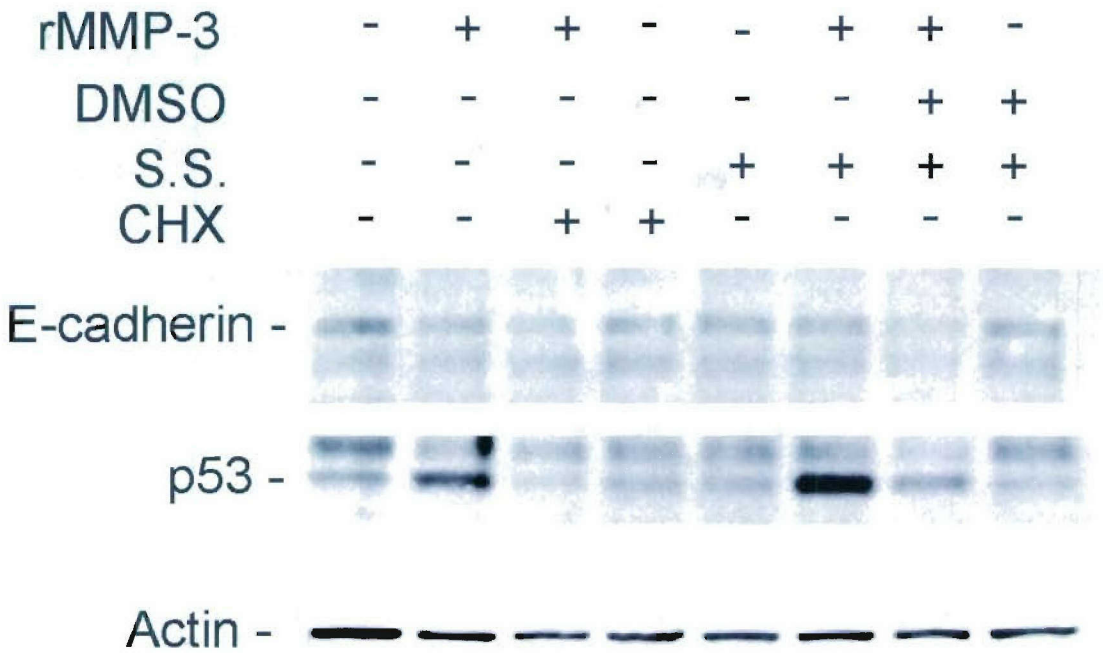



Fig 2. Mouse EPH4 mammary epithelial cells treated with MMP-3 show upregulation of p53. The ROS scavenger, DMSO, suppresses MMP-3-mediated upregulation of p53. Cyclohexamide, an inhibitor of translation also suppresses MMP-3-mediated p53 upregulation.

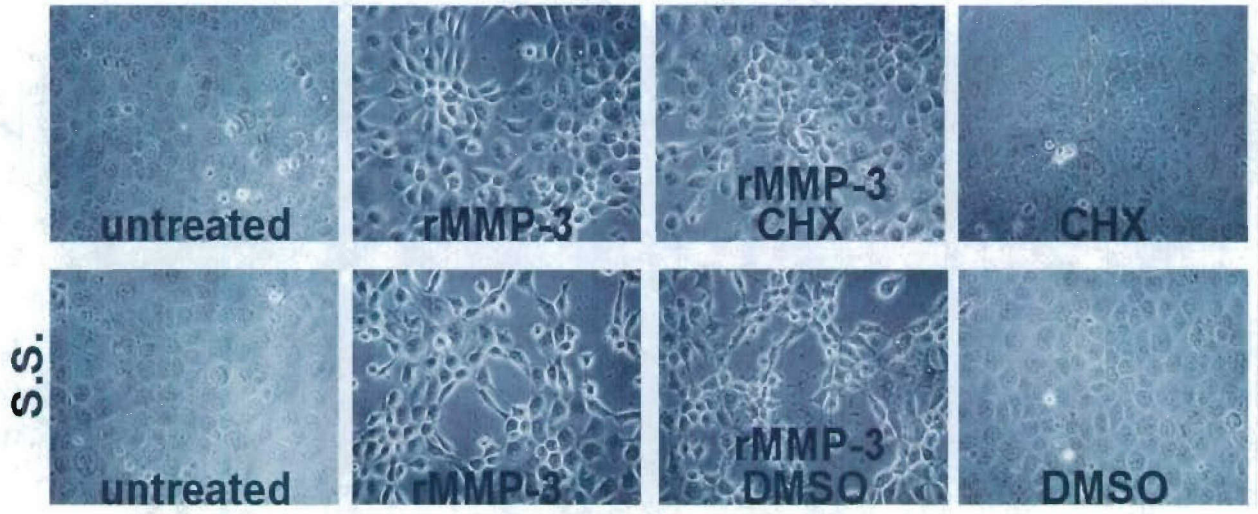



Fig. 3. Mouse EPH4 mammary epithelial cells undergo cell-cell detachment and cell rounding in response to MMP-3. DMSO and cyclohexamide (CHX), which suppresses p53 upregulation, fails to inhibit MMP-3-mediated cellular phenotypes. S.S is serum starved for 24 hrs prior to addition of MMP-3

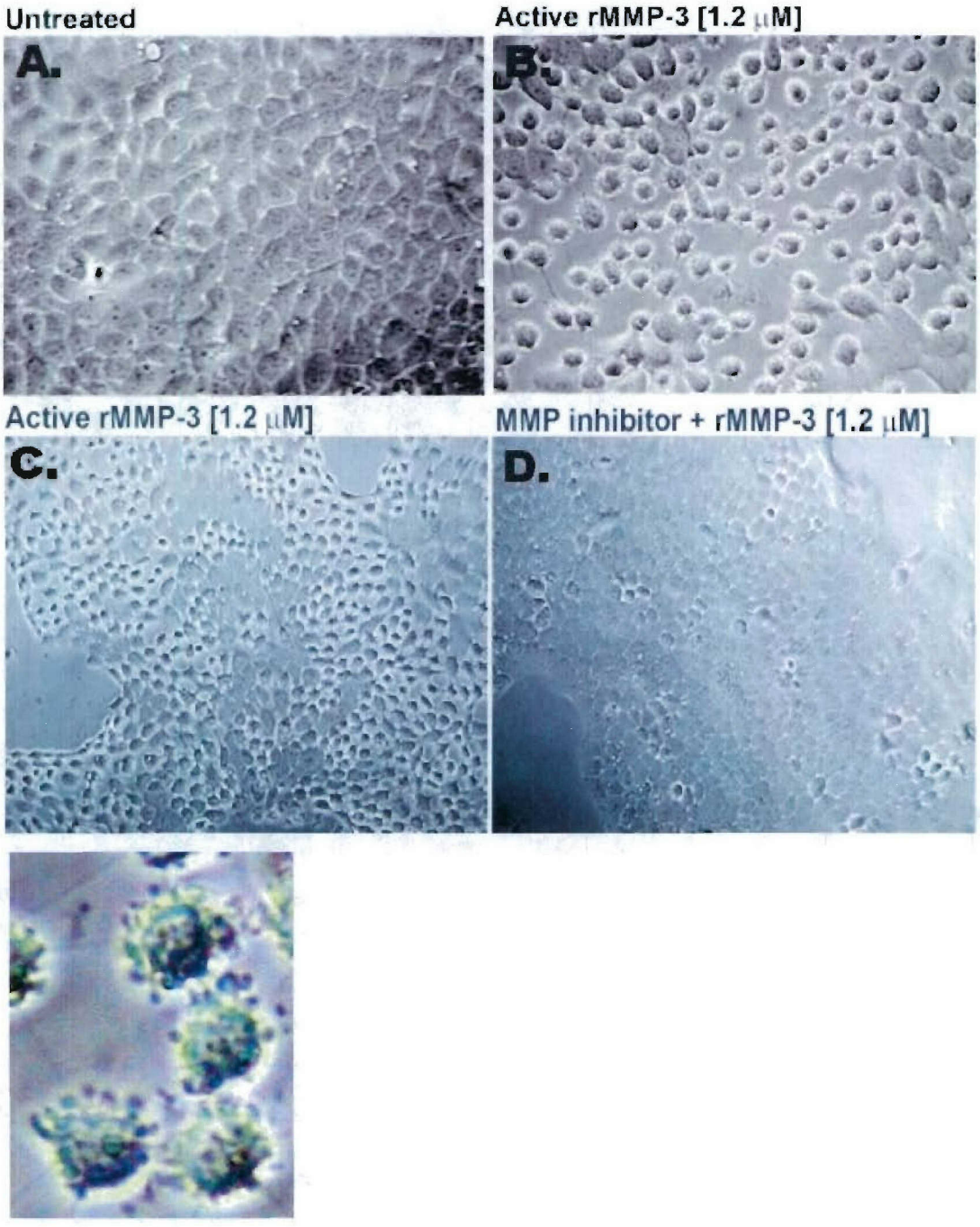



Fig. 4. High levels of MMP-3 (1.2 μM) induced cell-cell detachment and cell blebbing in MCF10A cells (B, C). This phenotype is suppressed with a broad spectrum MMP inhibitor (D). At high magnification, membrane blebbing is evident.

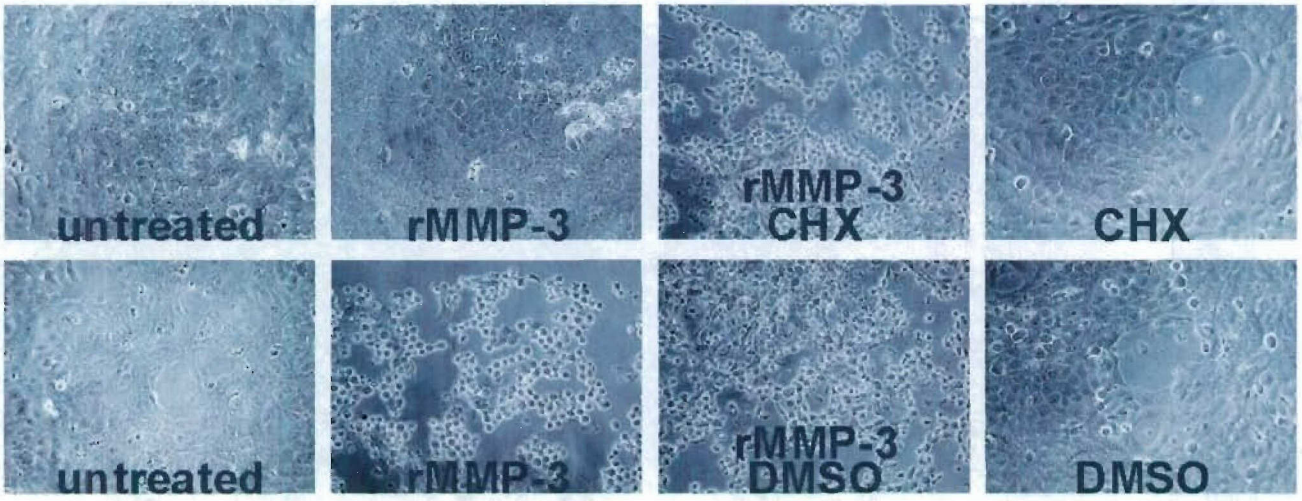



Fig. 5. MMP-3-mediated cell-cell detachment and cellular blebbing in MCF10A cells occurs only in serum starved cells or cells treated with cyclohexamide (CHX). Dimethyl sulfoxide does not alter the phenotype. The concentration of MMP-3 is 1.2μM.

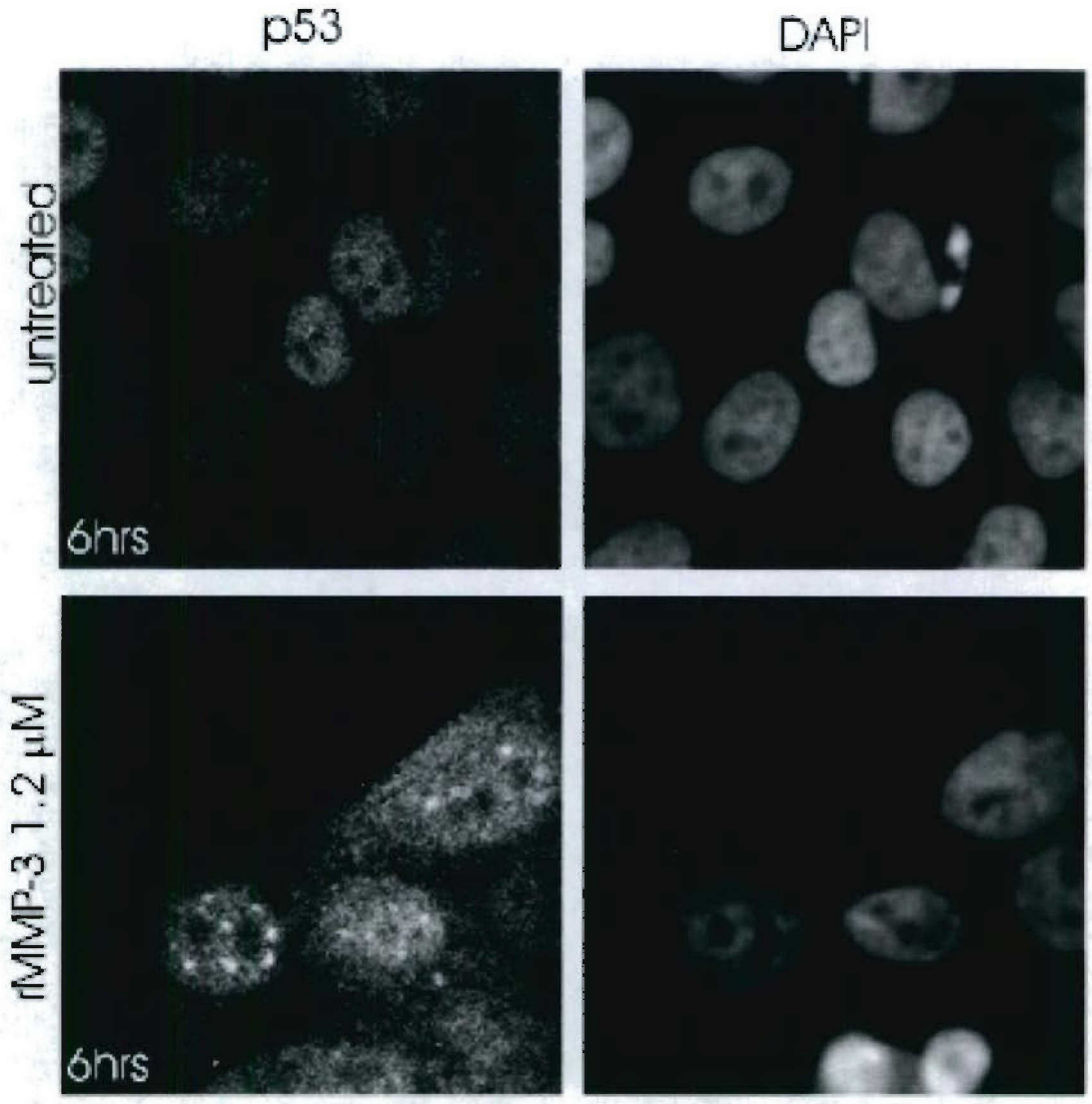



Fig. 6. P53 immunolocalization after MMP-3 treatment. Confocal images of p53 reveal increased nuclear and cytoplasmic staining and sub-nuclear foci in MCF10A cells treated with MMP-3 for 6 hrs.

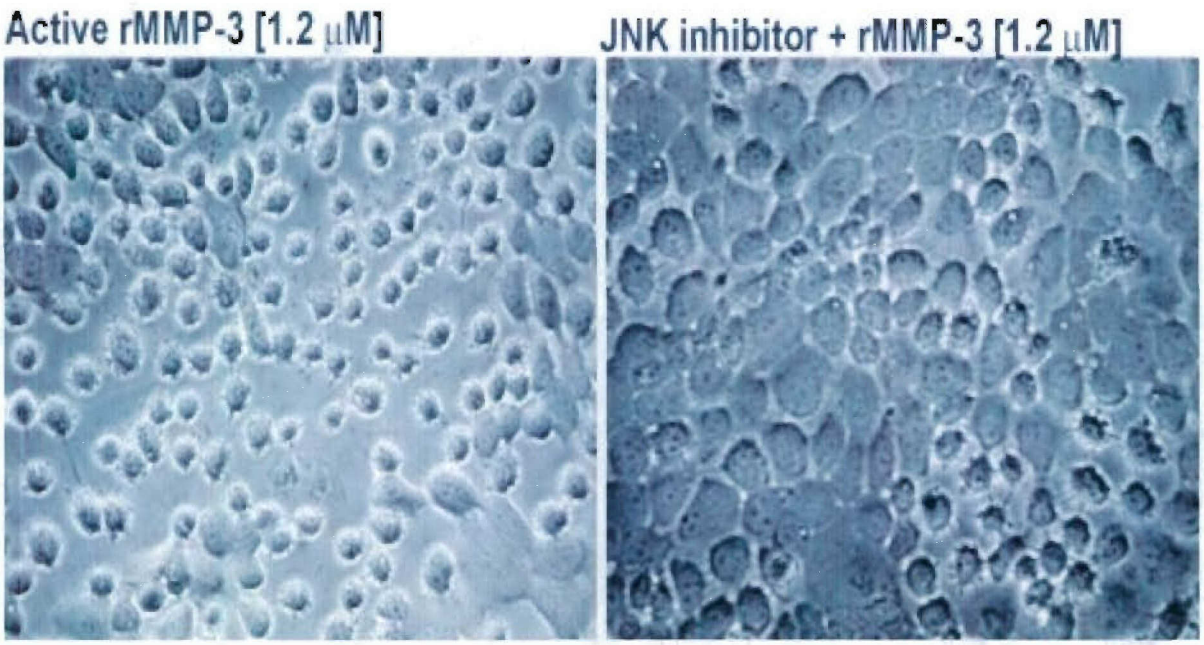
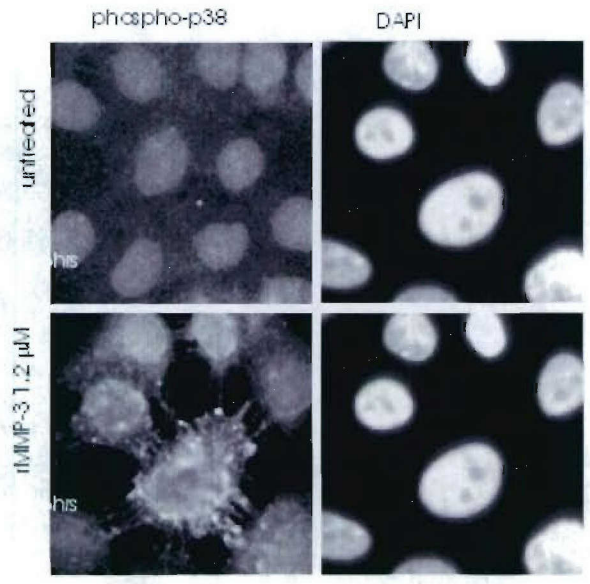



Fig. 7. MMP-3 mediated cell-cell detachment and blebbing in MCF10A cells is suppressed by a JNK inhibitor (10 μM). JNK inhibitor alone is similar to untreated cells (data not shown).

A.

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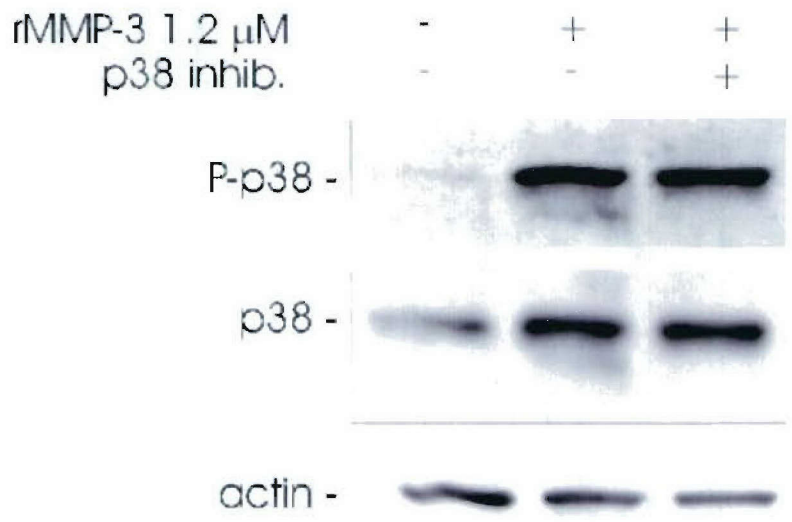



Fig. 8A. Immunolocalization of activated p38 using confocal imaging. MCF10 cells treated with MMP-3 exhibit increase staining for the stress activated kinase p38.

Fig. 8B. Active p38 (P-p38) is upregulated in cell lysates treated with MMP-3. As expected, the inhibitor against active p38 suppresses activity (data not shown) but does not suppress phosphorylation. Total levels of p38 are also increased in MMP-3 cells.

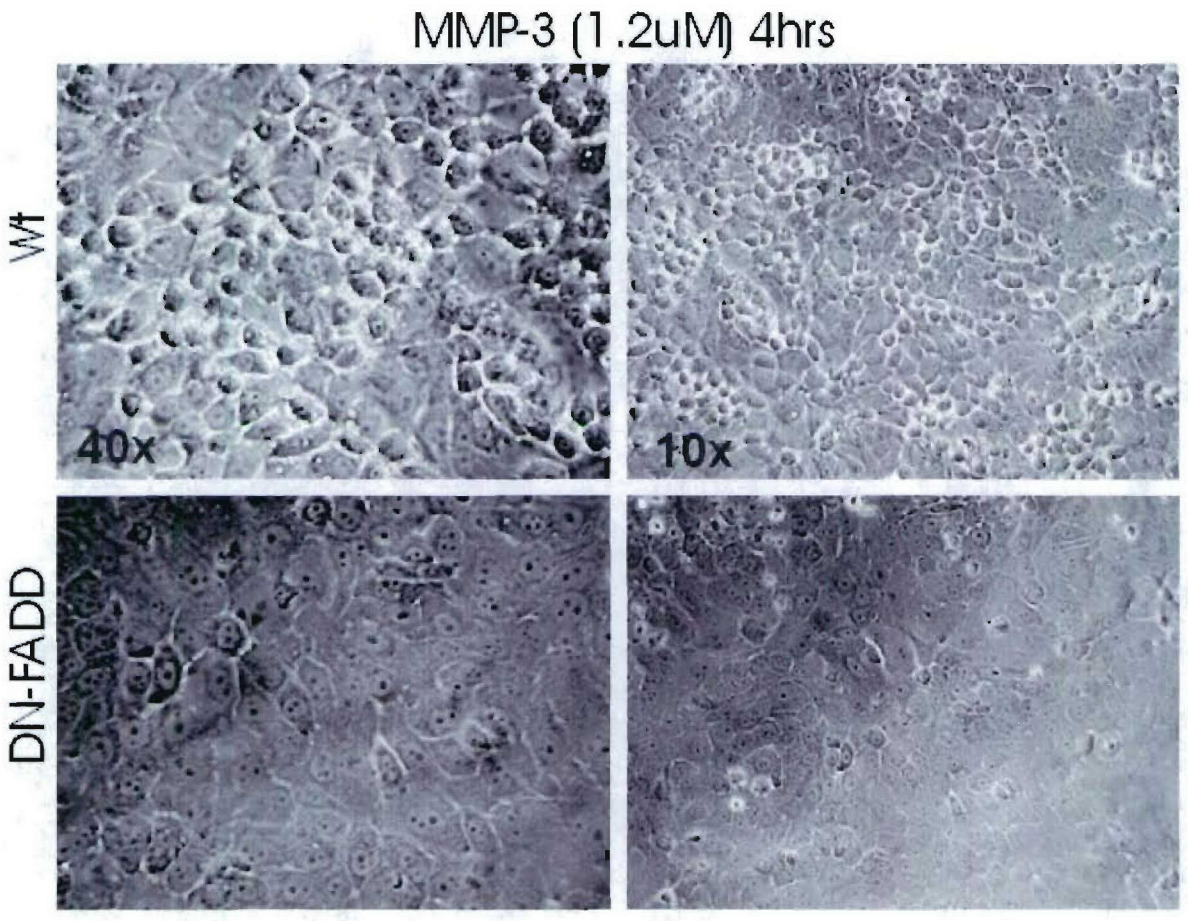



Fig. 9. Stable expression of a dominant negative FADD (DN-FADD) in MCF10A cells completely suppresses MMP-3 mediated cell-cell detachment and blebbing.

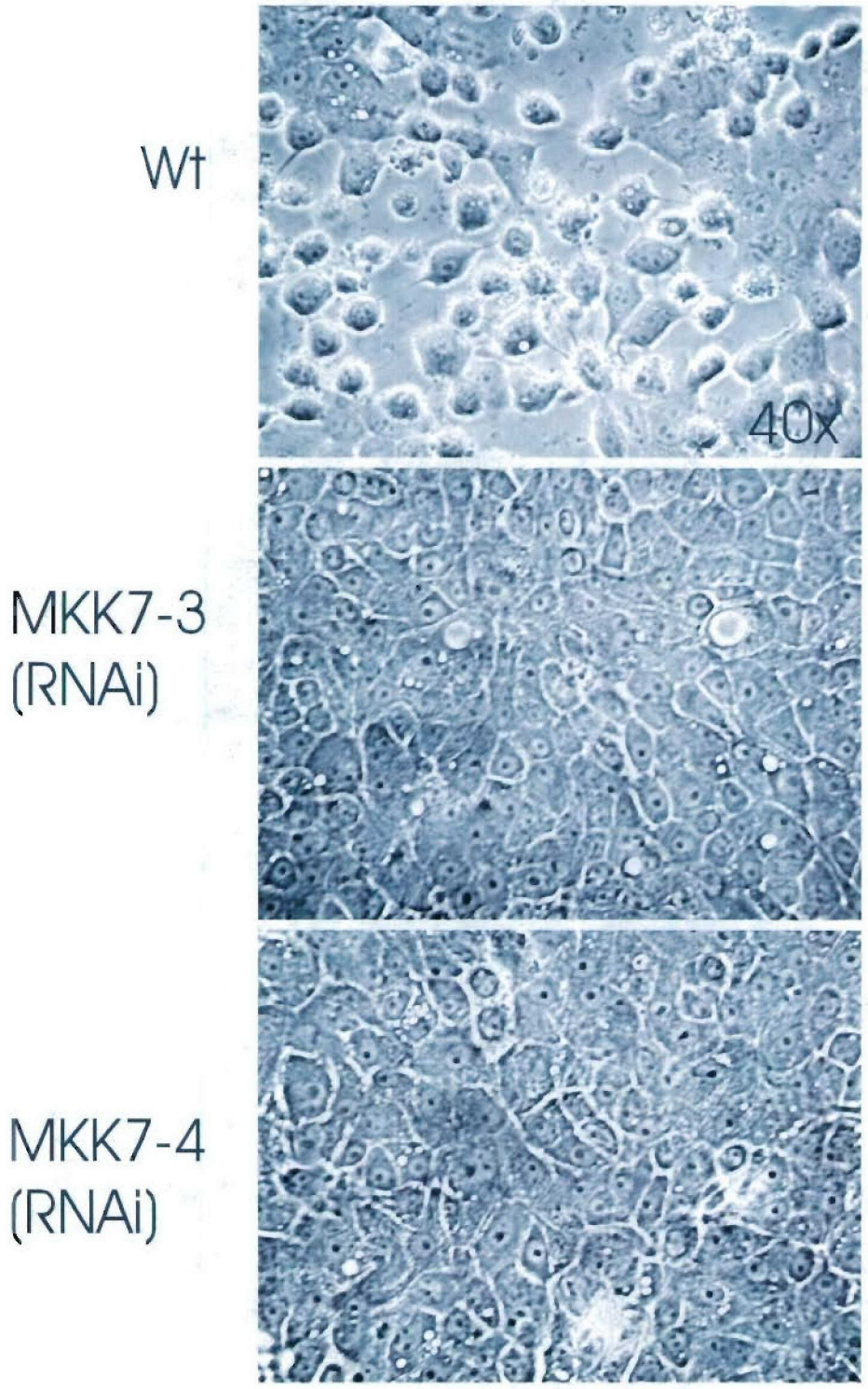
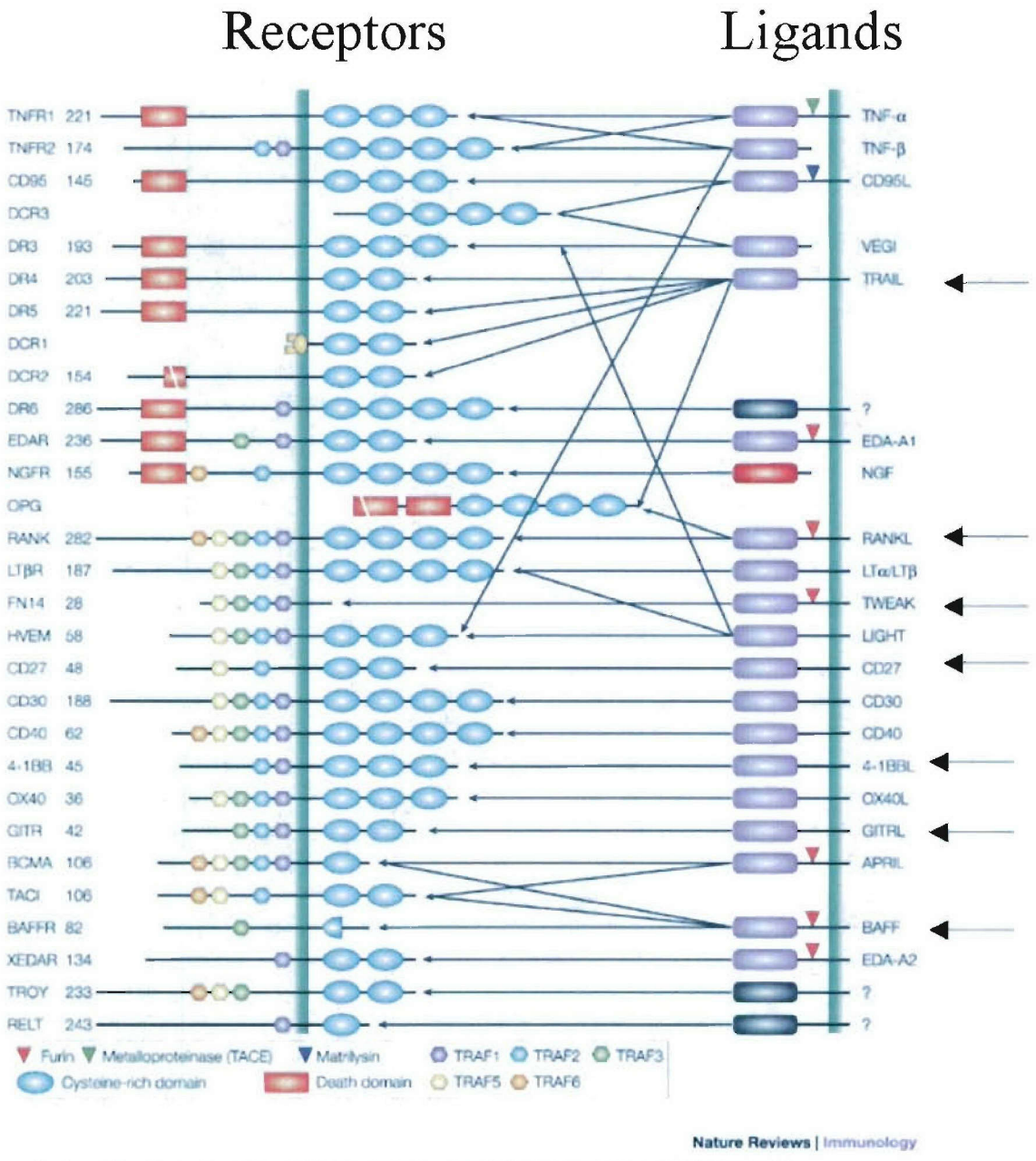



Fig. 10. MCF10A cells expressing RNAi against MKK7 are resistant to MMP-3-induced cell blebbing and cell-cell detachment.




*Nature Reviews Immunology* **3**; 745-756 (2003); doi:10.1038/nri1184
**SIGNALLING PATHWAYS OF THE TNF SUPERFAMILY: A DOUBLE-EDGED SWORD**

Fig. 11. RT-PCR reveals that MCF10 cells express a number of TNF superfamily ligands (arrows).

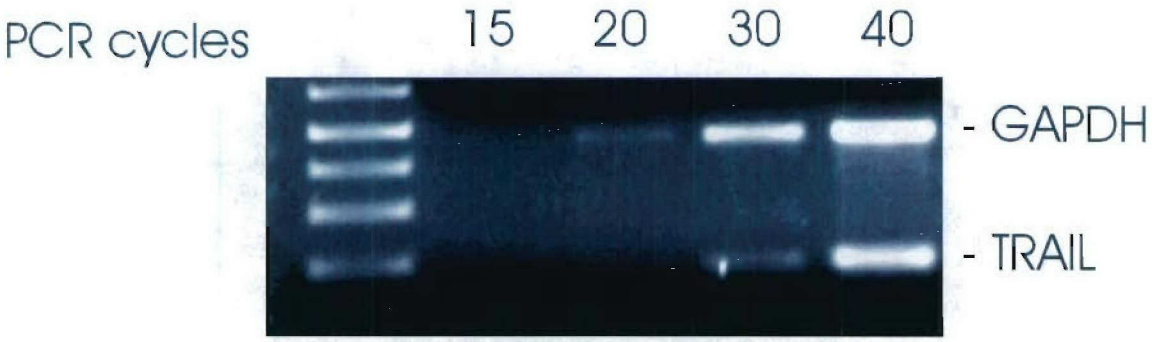



Fig. 12. TRAIL is expressed on MCF10A cells and is a possible candidate that could be shed and activated by MMP-3 activity.

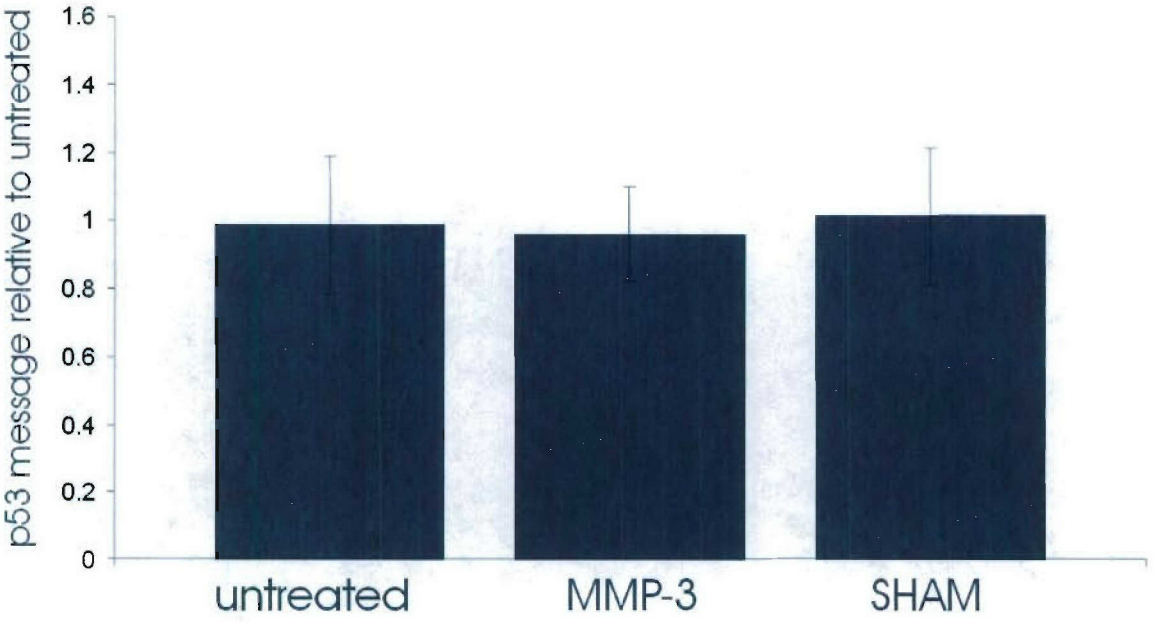



Fig. 13. MMP-3 treatment does not lead to upregulation of p53 message in mouse EPH4 mammary epithelial cells.

APPENDIX II

**ASCB**

Regulation of p53 in Mammary Epithelial Cells by MMP-3

Fata, JE[1], Pickering, C[2], Tlsty, TD[2], Werb, Z[3], and Bissell, MJ[1]

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Emerging evidence suggest that epigenetic pathways that modulate or compromise p53 function can contribute to the initial development of tumors. Overexpression of a secreted matrix metalloproteinase (MMP)-3 (stromelysin-1), in cultured mammary epithelial cells induces epithelial-to-mesenchymal transition (Lochter et al., JCB, 1997), and in vivo induces genetic instability and mammary tumors (Sternlicht et al., Cell, 1999). The mechanisms in which MMPs can cause these dramatic effects are not understood. We have now determined that MMP-3 can regulate the tumor suppressor protein, p53, and its target gene, p21, in mouse primary mammary epithelial cells and in normal human breast epithelial cells. Upon addition of recombinant MMP-3 (150 nM) to serum starved MCF10A cells, p53 protein began to decrease within 1 hr reaching < 50% by 6 hr. The loss of both p53 and p21, in response to MMP-3, was dose-dependent. By flow cytometry for DNA ploidy, quiescent cells treated with MMP-3 for 24 hr were primarily found within the G2/M phase, while the majority of untreated cells remained in G0/G1. MMP-3 also increased the population of S-phase cells. Our data supports the function of extracellular MMP-3 as an epigenetic factor capable of down-regulating p53 protein and p53 activity. One consequence of this down-regulation may be inappropriate entry of cells into the cell cycle during environmental stress.

Supported by grants from the OBER Office of the DOE, the NIH and The DOD Breast Cancer Research Program

**DOD ERA of Hope.**

Fata, JE[1], Werb, Z[2] and Bissell, MJ[1]

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ABSTRACT

Cell contraction and membrane blebbing are evolutionarily conserved events that occur during the execution phase of apoptosis. Several members of the TNF-ligand superfamily, which are associated with the promotion of a number of pathological processes, including inflammation and cancer are also capable of inducing membrane blebbing in some cell types. The majority of these ligands are transmembrane bound but can be shed from the cell surface through proteolytic processing where soluble ligands can act as antagonists, as in the case of FAS ligand, or agonists, as seen with TNF-α. Here we provide evidence that the matrix metalloproteinase, MMP-3/stromelysin-1 induces rapid membrane blebbing in serum starved or cyclohexamide-treated MCF10A human breast epithelial cells. MMP-3-mediated membrane blebbing is associated with reorganization of the actin cytoskeleton, upregulation of both p53 and p38 MAP kinase activity, and loss of cell surface E-cadherin. A broad-spectrum MMP inhibitor completely abolishes these reactions. To understand the signalling cascade initiated by MMP-3, we asked whether factors down-stream of TNF-superfamily signalling were involved. We show that inhibitors against JNK and caspase-3, and RNAi reduction of MKK7, a known activator of JNK inhibit membrane blebbing. Moreover, stable expression of a dominant negative FADD (dnFADD), a downstream effector of several TNF superfamily ligands, renders MCF10A cells resistant to membrane blebbing. Together these findings indicate that MMP-3 induces cell membrane blebbing through a TNF-superfamily signalling pathway and provides an impetus to further explore this protease in inflammation and cancer. J.E.F. (jefata@lbl.gov) is supported by the Dept. of Defence BCRP (DAMD17-03-1-0486). This work was also partially supported by the Dept. of Energy to M.J.B. (DE AC03 76SF00098).

**Publication**

*Nature* 436, 123-127.

Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability

Derek C. Radisky[1], Dinah D. Levy[1], Laurie E. Littlepage[2], Hong Liu[1], Celeste M. Nelson[1], Jimmie E. Fata[1], Devin Leake[3], Elizabeth L. Godden[3], Donna G. Albertson[4], M. Angela Nieto[5], Zena Werb[2] and Mina J. Bissell[1]

The tumour microenvironment can be a potent carcinogen, not only by facilitating cancer progression and activating dormant cancer cells, but also by stimulating tumour formation[1]. We have previously investigated stromelysin-1/matrix metalloproteinase-3 (MMP-3), a stromal enzyme upregulated in many breast tumours[2], and found that MMP-3 can cause epithelial–mesenchymal transition (EMT) and malignant transformation in cultured cells[3, 4, 5], and genomically unstable mammary carcinomas in transgenic mice[3]. Here we explain the molecular pathways by which MMP-3 exerts these effects: exposure of mouse mammary epithelial cells to MMP-3 induces the expression of an alternatively spliced form of Rac1, which causes an increase in cellular reactive oxygen species (ROS). The ROS stimulate the expression of the transcription factor Snail and EMT, and cause oxidative damage to DNA and genomic instability. These findings identify a previously undescribed pathway in which a component of the breast tumour microenvironment alters cellular structure in culture and tissue structure *in vivo*, leading to malignant transformation.